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Request for grant of a patent

The Patent Office
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1.	Your reference	8095GB/CAH/TJH
2.	Patent application number *(The Patent Office will fill in this)*	0205455.9 07 MAR 2002
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	MOLECULAR SENSING PLC 3 Challeymead Business Park Bradford Road Melksham SN12 8LH
	Patents ADP number *(if you know it)*	7830813001
	If the applicant is a corporate body, give the country/state of its incorporation	UNITED KINGDOM
4.	Title of the invention	NUCLEIC ACID PROBES, THEIR SYNTHESIS AND USE
5.	Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Abel & Imray 20 Red Lion Street London WC1R 4PQ
	Patents ADP number *(if you know it)*	174001 ✓

6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day/month/year)*

7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day/month/year)*

8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.*	Yes

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Description	37
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Translations of priority documents	
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Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
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11. I/We request the grant of a patent on the basis of this application.

Signature: Abel & Imray

Date: 7 March 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Dr Tim Hewson 01225 469 914

Molecular Sensing PLC

Case No. 8095

"Nucleic acid probes, their synthesis and use"

ABEL & IMRAY,
CHARTERED PATENT ATTORNEYS,
20 RED LION STREET,
LONDON, WC1R 4PQ

Nucleic acid probes, their synthesis and use

Field of the invention

The invention relates to probes for the sequence-specific detection of nucleic acids. The invention also relates to probes for the detection of nucleic acid binding proteins by virtue of their preferential binding to nucleic acids containing recognition sequences. More particularly the invention relates to labelled oligonucleotides suitable for use in the detection of nucleic acid and/or protein.

Background of the invention

The detection of specific DNA or RNA sequences is important for a wide range of applications within food, environmental and clinical diagnostics industries, and in the genomic, academic, pharmaceutical and pharmacogenetic research sectors. Detection methodologies should ideally be sensitive, sequence-specific, relatively rapid, low cost, accurate and suitable for routine use and/or automation. Further they should ideally be capable of being integrated with existing DNA amplification methodologies for example the polymerase chain reaction (PCR) and other nucleic amplification methodologies.

In addition to nucleic acid detection methods based on or integrated with amplification techniques such as PCR, there are also known techniques for sequence specific nucleic acid detection which are based on specific binding of a probe to a target which need not necessarily have been previously amplified. Southern and Northern blotting are known examples of such techniques.

Techniques that do not include an amplification stage must usually be highly sensitive in order to detect a signal. Typically autoradiography or chemiluminescence based techniques are used to produce the required sensitivity.

Southern and Northern blotting require the binding of the target nucleic acid to a membrane substrate. This requirement is disadvantageous because it is time consuming and poorly suited to automation.

Amplification based DNA detection methods normally utilize a range of fluorescence chemistries or radioactive labels. Frequently, target DNA to be analysed is amplified enzymically e.g. by PCR, and then visualized using a fluorescent DNA binding dye to stain DNA size-separated by gel electrophoresis. Alternative methods that do not require gel electrophoresis have been developed. These frequently allow real-time detection of DNA amplification with non-sequence-specific fluorescent dyes e.g. SYBR Green or ethidium bromide. Assays have also been developed that integrate DNA amplification by PCR with fluorescence-based detection using an expanding variety of fluorescently labelled oligonucleotide probes that hybridise to specific DNA sequences. A number of assays have been developed that utilize the nuclease activity of a DNA polymerase. Examples of commercially available nuclease assays include Invader (trade mark - Third Wave Technologies), Readit (trade mark - Promega) and TaqMan (trade mark - Applied Biosystems). In TaqMan assays described for example in patents US 5,487,972, US 5,538,848 and US 5,804,375 a hybridisation oligonucleotide is digested by the inherent 5' nuclease

activity of *Taq* polymerase concomitant to primer extension by the polymerase activity of *Taq*.

The application of electrochemistry to DNA detection offers potential advantages over other detection systems in terms of sensitivity and simplicity. Their portability, robustness, ease of miniaturization and potential for high volume manufacturing makes electrochemical methods especially suitable for clinical, food and environmental diagnostics.

The major focus for electrochemically-based gene probes has been on electrode-linked hybridisation techniques. Typically a capture probe (oligonucleotide or peptide nucleic acid) is immobilized on an electrode surface and it extracts the complementary target nucleic acid from a complex mixture of nucleic acids. The hybridisation event is transduced into a measurable electronic signal using either a redox-active hybridisation indicator (e.g. a ruthenium or cobalt salt), a redox-active indicator brought into contact with the target using a secondary probe, or by the direct measurement of changes in electrode capacitance caused by changes in the physical characteristics of the interface between the electrode and solution as a result of hybridisation. Frequently, these systems require prior amplification, e.g. by PCR, of the target sequence in order to achieve sufficient sensitivity.

Methods for detecting nucleic acid binding proteins include nuclease protection assays. In such assays a nucleic acid probe is mixed in solution with a putative nucleic acid binding protein. Under appropriate conditions nucleic acid binding proteins can be made to

bind to the nucleic acid sequence present in the probe. Following putative binding any unbound probe or region of probe can be digested by a suitable nuclease. Bound nucleic acid probe will be protected from nuclease digestion because the bound protein will sterically hinder the nuclease. Digested and undigested nucleic acid probe are then separated, for example by gel filtration, gel-electrophoresis or by encouraging undigested nucleic acid to bind to a membrane or other substrate, and quantified. Typically the probe is labelled with a radioactive isotope in order that it and its breakdown products can be quantified. There are drawbacks to using radioisotopes including problems with radioactive decay reducing the shelf life of reagents and occupational health and environmental concerns.

Nucleic acid probes suitable for detecting nucleic acid binding proteins include nucleic acids substantially of the sequence known to bind nucleic binding proteins *in vivo*. Additionally suitable probes for detecting nucleic acid binding proteins include aptamers which are nucleic acids evolved *in vitro* to perform a specific function (see - for example Brody and Gold, Reviews in Molecular Biology 9(1999) 324-329, Jäschke *et al*, Synlett 6 (1999) 825-833 and Griffith & Tawfik, Current Opinion in Biotechnology 11 (2000) 338-353 for details). Aptamers may be produced to bind to potentially any specific protein not just proteins ordinarily considered to be nucleic acid binding protein.

The use of the term "hybridise" in the context of nucleic acids in this specification will be understood to mean specific binding of a first nucleic acid to a second nucleic acid of complementary sequence. It will also be

understood that in order for hybridisation to occur the complementarity of nucleic acid sequences is not required to be total. Hybridisation includes complementary binding that includes base mis-match to the extent that such mis-match shall not materially reduce the efficiency of the methods described.

The invention provides a method of probing for a nucleic acid comprising contacting a nucleic acid solution with an oligonucleotide probe labelled with an electrochemically active marker, providing conditions at which the probe is able to hybridise with any complementary (target) sequence which may be present in the nucleic acid solution, selectively degrading either hybridised or unhybridised nucleic acid probe, and electrochemically determining information relating to the electrochemically active marker. The information relating to the marker is expediently used to derive information concerning the presence or absence of at least one nucleic acid species. Preferably the electrochemical techniques used to quantify relative proportions of degraded and non-degraded probe.

A number of methods of selectively degrading either hybridised or unhybridised nucleic acid probe are available. These include enzymatic methods or chemical treatments. Enzymes may be used to degrade a nucleic acid probe by digestion that results in cleavage of a phospho ester bond or cleavage of a saccharide or glycosidic bond.

S1 nuclease isolated from *Aspergillus orzae* or another suitable source, or an enzyme having a similar specificity may be used to selectively digest

unhybridised nucleic acid. The 5′ nuclease activity of *Taq* polymerase or a similar enzyme may be used to digest a nucleic acid probe which has hybridised at a position on the target between a pair of PCR primers. In that case the probe would be digested concomitant to primer extension.

The Invader (trade mark) system of Third Wave Technologies Inc. (see US 5,846,717, US 5,837,450, US 5,795,763 and US 5,614,402) provides a fluorogenic nucleic acid detection system that may be adapted for use with an alternative embodiment of the electrochemical detection system of the present invention as illustrated in Fig. 14a. Briefly, two short oligonucleotide probes are allowed to hybridise with the target nucleic acid. The probes are so designed that, whilst both are able to hybridise for at least part of their length to form a nucleic acid duplex, there is a region of sequence overlap between the two probes. This produces a specific structure which is recognized by the cleavase enzyme which cleaves one of the probes to release a "5′ flap" from the overlap region. An electrochemically active marker may be linked to the primer which yields the 5′ flap, preferably at or towards the 5′ end of that primer. The presence of the 5′ flap in the reaction mixture may be detected by electrochemical techniques. Particularly, the electrochemically labelled 5′ flap may be discriminated from the electrochemically labelled primer by virtue of the different length oligonucleotide portion of each respective molecule.

Alternatively and as illustrated in Fig 14b, the 5′ flap is not required to be linked to an electrochemically active marker. The release of the 5′ flap is detected by

an oligonucleotide recognition cassette which forms a nucleic acid triplex region which is also recognised and cleaved by cleavase enzyme. An electrochemically active marker may be linked to the recognition cassette so that cleavage of the recognition cassette results in the electrochemically active marker being linked to a fragment of the recognition cassette as opposed to the full length recognition cassette. The electrochemically labelled recognition cassette fragment may be discriminated from the electrochemically labelled full length recognition cassette by virtue of the different length oligonucleotide portion of each respective molecule.

The present invention is based on the observation that an electrochemically active marker such as metallocene exhibits different electrochemical characteristics depending on whether or not it is attached to an nucleotide and whether or not that nucleotide is incorporated into oligonucleotide or not, and the length of any such oligonucleotide.

The size and characteristics of a molecule to which an electrochemically active marker is attached may influence the perceived characteristics of the electrochemical marker for example, by influencing its rate of migration by diffusion or in response to an electric field.

The electrochemical activity of a marker may also be influenced by steric effects resulting from the presence of the molecule to which it is linked. For example, steric hindrance may prevent the marker from approaching an electrode and accepting or donating electrons.

If the marker is attached to an oligonucleotide then the secondary structure of the oligonucleotide (as largely determined by primary sequence) may influence the physical properties of that marker. For example, if the marker is attached to an oligonucleotide that contains self-complementary primary sequence then the resultant stem and loop secondary structure may sterically hinder the electrochemically active marker and reduce the signal obtained by voltammetry. It will be understood that digestion of the oligonucleotide may destroy or release the stem and loop structure and reduce or abolish its influence on the marker.

It will also be apparent that because the secondary structure of oligonucleotides is dependent on temperature, the effects which an oligonucleotide will have on an electrochemically active marker vary with temperature.

A person skilled in the art will be able to select an appropriate temperature at which to carry out the electrochemical technique of the invention in order to achieve an optimum signal to background noise ratio for the technique. If the technique is incorporated into a PCR reaction or other technique for which a thermal cycling apparatus is used, measurement at a desired temperature may simply be made at an appropriate point in the PCR temperature regime.

In one form of method according to the invention PCR takes place concomitant to 5′ nuclease digestion of the probe labelled with an electrochemically active marker. It will be apparent that such method includes a real time PCR method in which the electrochemical activity of the

solution is automatically measured during or following each PCR cycle. As discussed above, the temperature (PCR phase) at which measurements are made may influence the quality of signal obtained.

For simplicity, the present invention has largely been described in terms of detecting a single nucleic acid species. It will, however, be appreciated that the invention includes a "multiplex" system by which the methods and apparatus disclosed may be used to detect more than one nucleic acid species simultaneously. An example of such a multiplex would be the use of oligonucleotide probes which are complementary to two or more different targets. Those probes might be distinguished from each other by being labelled with electrochemically active markers having different redox characteristics and therefore being separately identifiable by any suitable electrochemical technique for example, differential pulse voltammetry.

The invention also provides apparatus arranged to carry out any one or more of the methods disclosed herein. Such apparatus may include suitable electrodes, electrochemical cells, disposable plastic ware and apparatus for detecting, recording, manipulating and displaying results, and in the case of PCR methods, appropriately programmed or programmable thermal cyclers. Such apparatus may also include apparatus for the optimal design of primers, probes and cycling conditions.

The labelled oligonucleotides used in accordance with a first aspect of the invention are capable of producing a distinct or enhanced electrochemical signal due to the release of ferrocenylated mononucleotide, dinucleotide or

oligonucleotide from a hybridisation oligonucleotide in a sequence-dependent nuclease assay. Those assays depend on a nuclease activity to cause a change to the probe such that a novel or enhanced signal is produced on recognition of a specific nucleic acid sequence.

If desired, the electrochemical detection step may be carried out using one or more electrodes covered by a membrane which is selectively able to exclude molecules based on one or more characteristics, for example, characteristics selected from size, charge and hydrophobicity. That may assist in eliminating background current arising from, for example, charged nucleic acid or undigested labelled oligonucleotide.

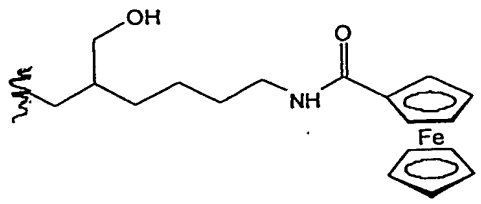
Ferrocenyl labels used in the probes according to the invention are advantageously N-substituted ferrocene carboxamides. The ferrocene ring, which constitutes the labelling moiety, may be unsubstituted. If desired, the ferrocene ring may be substituted by one or more substituents, the nature and location of which are selected so as to influence in a desired manner the redox characteristics of the ferrocene moiety. The ferrocene ring may additionally or instead be substituted by any ring substituents that do not materially reduce the electrochemical sensitivity of the label. The ferrocene carboxamide moiety may be linked via the carboxamide nitrogen to the nucleotide or oligonucleotide. Linkage to the nucleotide or oligonucleotide is preferably via a phosphate group or via the base of the nucleotide. Both methods of linkage permit the label to be attached via any nucleotide along the length of the oligonucleotide. However if linkage is via a phosphate group it is advantageously via a 3′ or 5′ terminal phosphate group so

as to minimise the likelihood that such linkage will sterically hinder Watson-Crick hybridisation of the oligonucleotide or effect nuclease activity. Linkage via a region of the base not involved in Watson-Crick base pairing is predicted to be less disruptive of such base pairing. Therefore linkage via the base maybe more suitable for labelling at non-terminal oligonucleotide sites. The label oligonucleotide may have a linker moiety between the oligonucleotide and the labelling moiety. Preferably, the labelled oligonucleotides have a ferrocenyl labelling moiety which is linked to the oligonucleotide by a linker moiety.

There may be used any suitable linker moiety. Suitable linker moieties may comprise an aliphatic chain which may be linear or branched, and saturated or unsaturated. Advantageously, the linker moiety is a linear or branched aliphatic chain having from 4 to 20 carbon atoms, and preferably from 6 to 16, especially from 8 to 14 atoms, especially 12 carbon atoms. The alkylene chains may be substituted by any substituent or may be interrupted by any atom or moiety provided that any such substituent, atom or moiety does not materially reduce the electrochemical sensitivity of the label. Illustrative of the ferrocenyl labels which may be used in accordance with the invention are those in Formulae I to III. Formula IV is illustrative of a ferrocenyl label which may be attached via a nucleotide base, the amino-modified thymine base being included in Formula IV for the purposes of illustration.

The ferrocene labelled probes may be made by any suitable method. By way of example, the oligonucleotide may be modified by introduction of a radical having a terminal

amino group. Illustrative of such amino-modified nucleotides is the modified nucleotide of Formula V. The ferrocene may then be incorporated by reaction of the amino-modified nucleotide with the N-hydroxy-succinimide ester of ferrocene carboxylic acid (Formula VI) to obtain ferrocene labelled oligonucleotide.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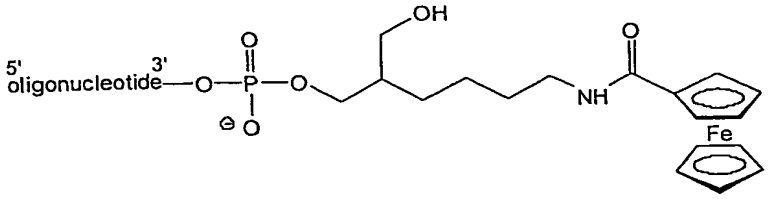
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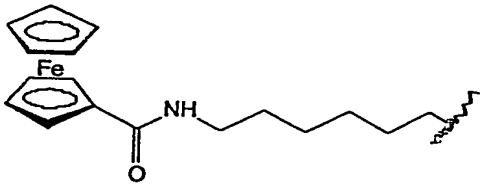
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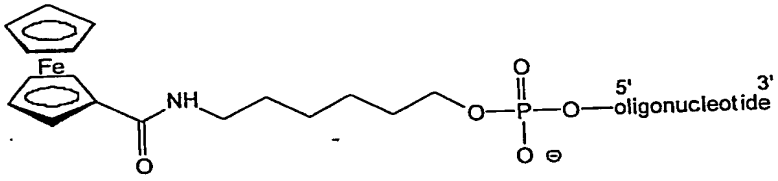
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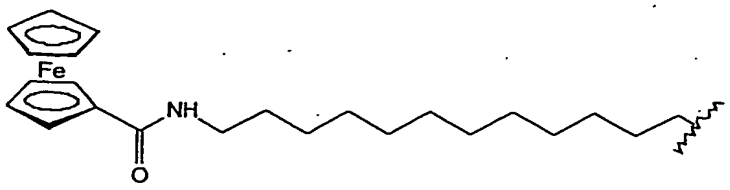

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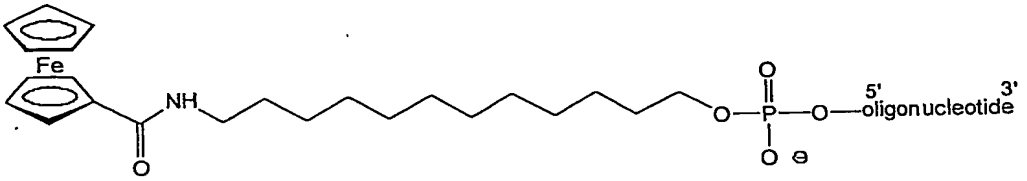
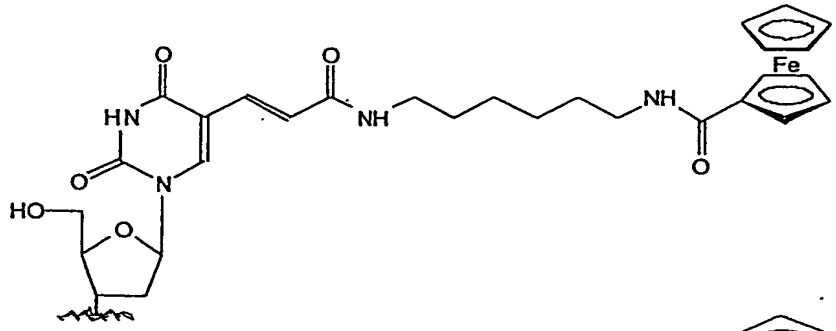
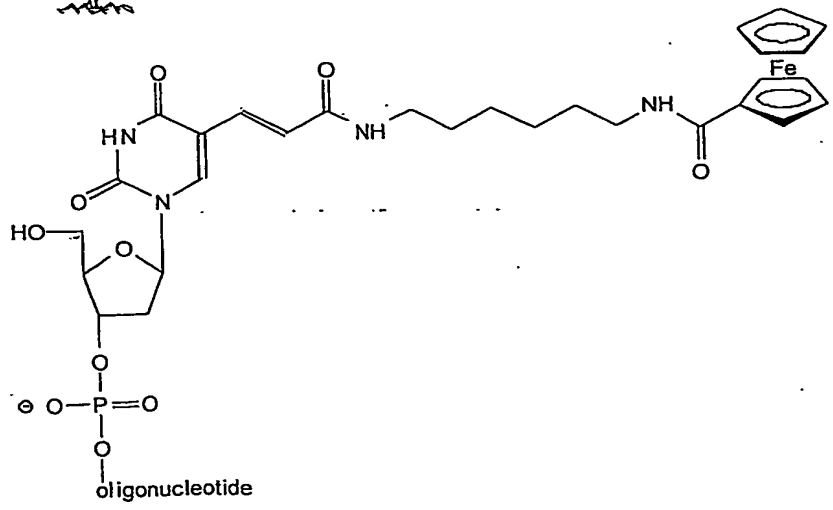


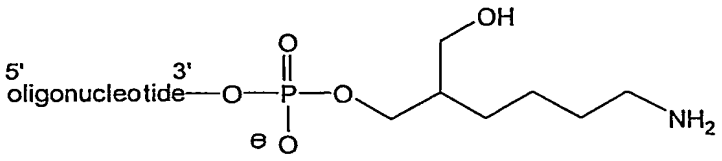
IIIa



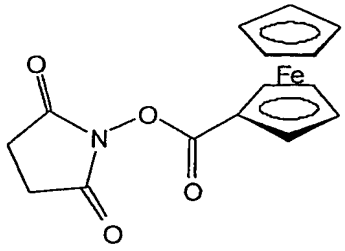
IV



IV a



V



VI

Certain illustrative embodiments of the invention will now be described in detail with reference to the accompanying drawings in which:

Fig. 1 is a schematic representation of an electrochemical cell used in differential pulse voltammetry measurements described herein;

Figs. 2a, 2b, 2c and 2d are differential pulse voltammograms of ferrocene labelled BAPR oligonucleotide as described in Example 4(a) below;

Figs. 3a, 3b, 3c and 3d are differential pulse voltammograms of ferrocene labelled BAPR oligonucleotide as described in Example 4(b) below;

Figs. 4a, 4b, 4c and 4d are differential pulse voltammograms of ferrocene labelled T1BAPR oligonucleotide as described in Example 4(c) below;

Figs. 5a 5b, 5c and 5d are differential pulse voltammograms of ferrocene labelled BAPR oligonucleotide as described in Example 4(d) below;

Figs. 6a, 6b, 6c and 6d are differential pulse voltammograms of ferrocene labelled GSDPR oligonucleotide as described in Example 4(e) below;

Figs. 7a, 7b, 7c and 7d are differential pulse voltammograms of ferrocene labelled MC11PR oligonucleotide as described in Example 4(f) below;

Figs. 8a and 8b are differential pulse voltammograms of unlabelled BAFR oligonucleotide as described in Example 4(g) below;

Figs 9a and 9b are differential pulse voltammograms of control reactions for ferrocene labelled T1BAPR oligonucleotide as described in Example 4(h) below;

Figs. 10a, 10b, 10c and 10d are differential pulse voltammograms of PCR mixture containing labelled BAPR oligonucleotide as described in Example 5(a) below;

Figs. 11a, 11b and 11c are differential pulse voltammograms of another PCR mixture containing ferrocene labelled BAPR oligonucleotide as described in Example 5(b) below; and

Figs. 12a, 12b, 12c and 12d are differential pulse voltammograms of a PCR mixture containing ferrocene labelled T1BAPR oligonucleotide as described in Example 5(c);

Figs. 13a, 13b, 13c and 13d are differential pulse voltammograms of a PCR mixture containing ferrocene labelled GSDPR oligonucleotide as described in Example 5(d);

Fig. 14a and 14b are schematic representations of the Invader fluorogenic nucleic acid detection system adaptated for use in a method of the invention.

With reference to Fig. 1, an electrochemical cell 1 suitable for use in the cyclic voltammetry experiments described herein comprises a vessel 2, containing a

background electrolyte solution 3, which is an aqueous 100mM solution of ammonium acetate. Immersed in the solution 3 is a chamber 4, which receives both the sample to be tested and, immersed therein, a glassy carbon working electrode 5. Also immersed in the solution 3 is a counter electrode 6 of platinum wire and a silver/silver chloride reference electrode 7 immersed in 4M potassium chloride solution, which solutions are in communication with others via a sintered disc.

The following Examples illustrate the invention:

Materials and methods - Oligonucleotide preparation and assays

Oligonucleotides were obtained from Sigma Gensosys. All oligonucleotides were obtained desalted and were used without further purification. N,N'-Dimethylformamide (DMF) (99.8% A.C.S. reagent) and zinc acetate dihydrate (99.999%) were obtained from Aldrich

Potassium bicarbonate (A.C.S. reagent), potassium carbonate (minimum 99%), ammonium acetate (approximately 98%), magnesium acetate (minimum 99%), ammonium persulfate (electrophoresis reagent), N,N,N',N'-tetramethylethylenediamine (TEMED) and molecular biology grade water were obtained from Sigma.

NAP10 columns (G25 DNA grade Sephadex trade mark) were obtained from Amersham Biosciences.

S1 Nuclease, dNTPs and human genomic DNA were obtained from Promega.

AmpliTaq Gold, with 25 mM magnesium chloride and GeneAmp (trade mark) 10X PCR Gold buffer supplied, was obtained from Applied Biosystems.

Incubations were performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc.). Absorbance measurements at 260nm were performed using a Cary 100 Bio spectrophotometer (Varian Ltd.).

Polyacrylamide gels were prepared with ProtoGel (National Diagnostics) and stained with SYBR Gold (Molecular Probes Inc.).

Agarose gels were prepared with SeaKem LE agarose (BioWhittaker Molecular Applications) and stained with ethidium bromide (Aldrich). Gels were electrophoresed in 0.5X Tris/borate/EDTA (TBE) buffer (Sigma). All solutions were prepared with autoclaved deionised water (WaterPro system, Labconco).

Oligonucleotide sequences

The oligonucleotide sequences of the glucose-6-phosphatase and medium chain acyl-CoA dehydrogenase primers and probes were as disclosed in Kunihior Fujii, Yoichi Matsubara, Jun Akanuma, Kazutoshi Takahashi, Shigeo Kure, Yoichi Suzuki, Masue Imiazumi, Kazuie Iinuma, Osamu Sakatsume, Piero Rinaldo, Kuniaki Narisawa; Human Mutation; 15; 189-196; (2000).

The oligonucleotide sequence of the beta actin primers and probe were as disclosed in Agnetha M Josefsson, Patrik K E Magnusson, Nathelie Ylitalo, Per Sorensn, Pernialla Qwarforth-Tubbin, PerKragh Andersen, Mads Melbye, Hans-Olov Adami, Ulf B Gyllensten; Lancet; 355; 2189-2193; (2000).

1. ACTB (β actin)

Probe

BAPR: ATG CCC TCC CCC ATG CCA TCC TGC GT

C9-T1BAPR: T(C9)G CCC TCC CCC ATG CCA TCC TGC GT

(T(C9) = amino modified thymine with C9 linker, Formula IV)

Primers
BAF: CAG CGG AAC CGC TCA TTG CCA ATG G
BAR: TCA CCC ACA CTG TGC CCA TCT ACG A
BAFR: CAG GTC CCG GCC AGC CAG

2. CFTR (cystic fibrosis transmembrane conductance regulator)
Primers
CFT01: AGG CCT AGT TGT CTT ACA GTC CT
CFT03: TGC CCC CTA ATT TGT TAC TTC

3. G6PC (glucose-6-phosphatase)
Probe
GSDPR: TGT GGA TGT GGC TGA AAG TTT CTG AAC
Primers
GSDw: CCG ATG GCG AAG CTG AAC
GSDcom: TGC TTT CTT CCA CTC AGG CA

4. ACADM (medium chain acyl-CoA dehydrogenase)
Probe
MC11PR: CTA GAA TGA GTT ACC AGA GAG CAG CTT GG
Primers
MC11w: GCT GGC TGA AAT GGC AAT GA
MC11com: CTG CAC AGC ATC AGT AGC TAA CTG A

Materials and Methods - Electrochemical Detection.

The following electrodes and low volume cell were obtained from BAS, Congleton, Cheshire, UK:

Glassy carbon working electrode (catalogue number MF-2012)

Silver/silver chloride reference electrode (catalogue number MF-2079)

Platinum wire counter (auxiliary) electrode (catalogue number MW-4130).

Low volume cell (catalogue number MF-2040) comprising glass voltammetry vial and glass sample chamber, with replaceable vycor tip.

An AutoLab electrochemical workstation (either PGSTAT30 with frequency response analyzer or μAutoLab type II manufactured by Eco Chemie B.V) was obtained from Windsor scientific Limited.

EXAMPLE 1

This Example describes the cyclic voltammetry method used in Examples 2 to 11 below.

The low volume cell of Fig. 1 was filled with approximately 10ml ammonium acetate solution (100mM).

A 200µl aliquot of the sample for analysis was placed in the glass sample chamber 4 which was then placed in the low volume cell along with the reference 7 and counter electrodes 6. The electrodes were connected to an Autolab electrochemical workstation and differential pulse voltammetry carried out using the parameters described below. Prior to analysis the glassy carbon working electrode was polished (using BAS polishing kit catalogue number MF-2060) followed by conditioning. Electrode conditioning consisted of cyclic voltammetry, sweeping between +/- 1 volt in the appropriate background buffer.

Table 1 Parameters for different pulse voltammetry:

Parameter:	Cathodic Sweep	Anodic Sweep
Conditioning potential (V)	0	0
Conditioning duration (s)	0	0
Deposition potential (V)	0.8	-0.1
Deposition duration (s)	5	5
Equilibration time (s)	0	0
Modulation time (s)	0.02	0.02
Interval time (s)	0.1	0.1
Initial potential (V)	0.75	-0.1
End potential (V)	0.1	0.7
Step potential (V)	0.005	0.005
Modulation amplitude (V)	0.1	0.1

EXAMPLE 2 - Synthesis of *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid

Ferrocenecarboxylic acid (303mg, 1.32mmol) and *N*-hydroxysuccinimide (170mg, 1.47mmol) were dissolved in dioxane (15ml) and added with stirring to a solution of dicyclohexylcarbodiimide (305mg, 1.48mmol) in dioxane (3ml). The mixture was stirred at room temperature for 24 hours during which time a precipitate was formed. The precipitate was removed by filtration, solvent was removed from the filtrate *in vacuo* and the resulting solid purified by silica gel column chromatography, eluting with 8:2 petrol:ethyl acetate. Yield 320mg, 74%.

EXAMPLE 3 - Synthesis of ferrocenyl oligonucleotides

Lyophilised amino-modified oligonucleotide was rehydrated in the correct volume of $K_2CO_3/KHCO_3$ buffer (500mM, pH 9.0) to give an oligonucleotide concentration of $0.5 nmol\mu l^{-1}$. Amino-modified oligonucleotide (40µl, $0.5 nmol\mu l^{-1}$) was added slowly with vortexing to a solution of the *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid in DMF (40µl, 375mM). The solution was shaken at room temperature overnight. It was then diluted with ammonium acetate (920µl, 100mM, pH 7.0) and purified using two NAP 10 columns, eluting firstly with ammonium acetate (100mM, pH 7.0), and then with autoclaved deionised water. Ferrocenylated oligonucleotides were partially purified by NAP 10 column to remove salt and low molecular weight ferrocene species to give a mixture of ferrocene labelled and unlabelled oligonucleotides. No further purification was carried out before use. Amino-modified oligonucleotides possessing four different linker structures: C7, C6, C12 and T(C9), varying in

structure and point of attachment, were used in labeling reactions. C6, C12 and T(C9) linkers were attached at the 5′ end of the oligonucleotide, via the terminal phosphate ester or the base. The C7 linker was attached via the terminal phosphate ester at the 3′ end of the oligonucleotide. The label structures are given in Formulae I to IV. Oligonucleotide concentration of the eluent was determined by measuring its absorbance at 260nm. Presence of the ferrocene label was confirmed by voltammetric analysis.

EXAMPLE 4

S1 Nuclease digestion

Oligonucleotide digestion reactions (100µl) contained oligonucleotide (3.5-9 µM, concentrations detailed below), ammonium acetate (250mM, pH 6.5), zinc acetate (4.5mM) and S1 Nuclease (0.4Uµl^{-1}). Reactions were incubated at 37°C for 1 hour. Complete digestion of the oligonucleotide was confirmed by polyacrylamide gel analysis of a 10µl aliquot of the crude reaction mix. Multiple reactions were pooled prior to voltammetric analysis, to give a final volume of 200µl. By way of comparison, "no-enzyme" reactions were performed as described above, omitting S1 Nuclease from the reaction mixture. Heated enzyme controls were performed as described above, using S1 Nuclease that had previously been thermally denatured by heating at 95° C for 15 minutes.

In the following, the reactants and conditions are as described above, and the voltammetry conditions are as given in Table 1 except where otherwise stated.

Example 4(a):

Oligonucleotide: BAPR oligonucleotide labelled at 3' end by ferrocene with a 7-carbon spacer moiety (Formula I).

Concentrate of oligonucleotide: 7.0µM

Voltammetry conditions: As in Table 1 except that the interval time was 0.09s and the modulation time 0.5s.

The results are shown in Fig. 2a (cathodic sweep of "no-enzyme" control), Fig. 2b (cathodic sweep of solution including S1 nuclease), Fig. 2c (anodic sweep of "no-enzyme" control) and 2d (anodic sweep of solution including S1 nuclease). The measured peak values, peak positions and % peak enhancement for the solution including S1 nuclease (that is, with digested oligonucleotide) as compared the "no-enzyme" control are given in Table 2.

Example 4(b):

Oligonucleotide: BAPR oligonucleotide labelled at 5′ end by ferrocene with a 6-carbon spacer moiety (Formula II).

Concentration of oligonucleotide: 7.0μM

Voltammetry conditions: As in Table 1 except that the interval time was 0.09s and the modulation time 0.5s.

The results are shown in Fig. 3a (cathodic sweep of "no-enzyme" control), Fig. 3b (cathodic sweep of solution including S1 nuclease), Fig. 3c (anodic sweep of "no-enzyme" control) and 3d (anodic sweep of solution including S1 nuclease). The measured peak values, peak positions and % peak enhancement for the solution including S1 nuclease (that is, with digested oligonucleotide) as compared the "no-enzyme" control are given in Table 2.

Example 4(c):

Oligonucleotide: T1BAPR oligonucleotide labelled at 3' end base by ferrocene with a 9-carbon spacer moiety (Formula IV).

Concentration of oligonucleotide: 8.8μM.

Voltammetry conditions: As in Table 1

The results are shown in Fig. 4a (cathodic sweep of "no-enzyme" control), Fig. 4b (cathodic sweep of solution including S1 nuclease), Fig. 4c (anodic sweep of "no-enzyme" control) and 4d (anodic sweep of solution including S1 nuclease). The measured peak values, peak positions and % peak enhancement for the solution including S1 nuclease (that is, with digested oligonucleotide) as compared the "no-enzyme" control are given in Table 2.

Example 4(d):

Oligonucleotide: BAPR oligonucleotide labelled at 5' end by ferrocene with a 12-carbon spacer moiety (Formula III).

Concentration of oligonucleotide: 7.0μM.

Voltammetry conditions: As in Table 1 except that the interval time was 0.09s and the modulation time 0.5s

The results are shown in Fig.5a (cathodic sweep of "no-enzyme" control), Fig. 5b (cathodic sweep of solution including S1 nuclease), Fig. 5c (anodic sweep of "no-

enzyme" control) and 5d (anodic sweep of solution including S1 nuclease). The measured peak values, peak positions and % peak enhancement for the solution including S1 nuclease (that is, with digested oligonucleotide) as compared the "no-enzyme" control are given in Table 2.

Example 4(e):

Oligonucleotide: GSDPR oligonucleotide labelled at 5' end by ferrocene with a 12-carbon spacer moiety (Formula III).

Concentration of oligonucleotide: 3.5μM.

Voltammetry conditions: As in Table 1.

The results are shown in Fig. 6a (cathodic sweep of "no-enzyme" control), Fig. 6b (cathodic sweep of solution including S1 nuclease), Fig. 6c (anodic sweep of "no-enzyme" control) and 6d (anodic sweep of solution including S1 nuclease). The measured peak values, peak positions and % peak enhancement for the solution including S1 nuclease (that is, with digested oligonucleotide) as compared the "no-enzyme" control are given in Table 2.

Example 4(f):

Oligonucleotide: MC11PR oligonucleotide labelled at 5' end by ferrocene with a 12-carbon spacer moiety (Formula III).

Concentration of oligonucleotide: 3.5μM.

Voltammetry conditions: As in Table 1.

The results are shown in Fig. 7a (cathodic sweep of "no-enzyme" control), Fig. 7b (cathodic sweep of solution including S1 nuclease), Fig. 7c (anodic sweep of "no-enzyme" control) and 7d (anodic sweep of solution including S1 nuclease). The measured peak values, peak positions and % peak enhancement for the solution including S1 nuclease (that is, with digested oligonucleotide) as compared the "no-enzyme" control are given in Table 2.

Example 4(g) (comparison):

Oligonucleotide: BAFR, unlabelled.

Concentration of oligonucleotide: 8.8μM.
Voltammetry conditions: As in Table 1

The results are shown in Fig. 8a (cathodic sweep) and Fig. 8b (anodic sweep). No peak was observed in either sweep.

Example 4(h) (Comparison):

Oligonucleotide: T1BAPR oligonucleotide labelled at 5' end base by ferrocene with a 9-carbon spacer moiety (Formula IV).

Concentration of oligonucleotide: 8.8μM.

Voltammetry conditions: As in Table 1.

The results are shown in Fig. 9a (anodic sweep of "no-enzyme" control) and Fig. 9b (anodic sweep of heated enzyme control including S1 nuclease). In Fig. 9a, a peak height of 60.6μA (peak position 424mV) was recorded, whilst in Fig. 9b, a peak height of 39.9μA (peak position 409mV) was recorded.

Ferrocene related peaks were observed at 300-500mV. No peaks were observed in this range when non-ferrocenylated oligonucleotides were analysed (Figs. 8a and 8b). Comparison of digested ferrocene labelled oligonucleotides and no-enzyme controls showed that an increase in peak height was obtained on digestion of the oligonucleotide (Table 2).

In order to confirm that the observed changes were not due to the presence of enzyme, or components of the enzyme storage buffer, digestion experiments were also performed using heat-denatured enzyme (Example 4(h)). No significant changes to the ferrocene signal were observed when comparing heat denatured enzyme and no enzyme controls.

Digestion experiments of two additional oligonucleotide sequences with the C12 ferrocene-oligonucleotide linker were performed; Ferrocene-C12-MC11PR and Ferrocene-C12-GSDPR (Figures 6 and 10). An increase in peak height of the ferrocene related signal of digested oligonucleotide was observed for each sequence.

Table 2 Positions and heights for ferrocene related peaks on anodic and cathodic differential pulse voltammograms

Cathodic Sweeps

	Undigested		Digested		
Oligo	Peak position	Peak Height	Peak Position	Peak Height	% increase in peak height upon digestion
BAPR C7	419	-4.65	424	-10.16	218
BAPR C6	424	-3.24	444	-8.87	274
T1BAPR C9	518	-94.1	533	-456.5	485
BAPR C12	-	-	500	-4.71	
GSDPR C12	533	-30.5	554	-65.43	215
MC11PR C12	553	-21.9	564	-49	224

Anodic Sweeps

	Undigested		Digested		
Oligo	Peak position	Peak Height	Peak Position	Peak Height	% increase in peak height upon digestion
BAPR C7	394	3.39	394	9.18	266
BAPR C6	399	1.63	419	10.3	632
T1BAPR C9	434	82.8	444	818	988
BAPR C12	-	-	494	6.7	
GSDPR C12	434	62.9	394	359	571
MC11PR C12	429	60.1	394	196	326

EXAMPLE 5 - PCR

PCR amplification was performed from human genomic DNA (40ng per 100µl reaction), or gel purified PCR amplicons. PCR amplicons used for subsequent amplifications were purified by agarose gel with Nucleospin Extract kits

(Macherey-Nagel) following the protocol supplied. All ferrocenyl oligonucleotide probes were 3′ phosphorylated.

Primers, template and probe used for individual reactions are detailed above.

100µl reactions contained Tris HCl (15mM, pH 8.0), potassium chloride (50mM), magnesium chloride (3.5mM), dATP, TTP, dCTP, dGTP (200µM each), forward primer (1.0µM), reverse primer (1.0µM), ferrocenyl oligonucelotide probe (0.9µM), AmpliTaq Gold (0.04 $Uµl^{-1}$). Samples were incubated at 95°C for 10 minutes (initial denaturation and enzyme activation) followed by 40 cycles of denaturation at 95°C for 15s, and primer annealing and extension at 60°C for 1 min.

Fifteen 100µl reactions were prepared and pooled. The crude reaction mixture was then concentrated to 200µl total volume prior to voltammetric analysis.

In the following, the reactants and conditions are as described above and the voltammetry conditions are as given in Table 1 unless otherwise stated.

Example 5(a):

Oligonucleotide: BAPR oligonucleotide labelled at 5′ end with a 12-carbon spacer moiety (Formula III).

Positive reaction: (β actin) template: β actin PCR amplicon; primers: BAF, BAR.

Negative reaction: (cystic fibrosis transmembrane conductance regulator) template: cystic fibrosis PCR amplicon; primers: CFT 01, CFT 03.

Voltammetry conditions: As in Table 1.

The results were as follows:

Figure 10a negative reaction, cathodic sweep, no peak observed

Figure 10b positive reaction, cathodic sweep, peak position: 493mV, peak height: -19.4nA.

Figure 10c negative reaction, anodic sweep, no peak observed.

Figure 10d positive reaction, anodic sweep, peak position: 373mV, peak height: 27,3nA.

Example 5(b):

Oligonucleotide: MC11PR oligonucleotide labelled at 5' end with a 12-carbon spacer moiety (Formula III).

Positive reaction: (Medium chain acyl-CoA dehydrogenase) template: MCAD PCR amplicon or genomic template; primers: MC11w, MC11com;
Negative reaction: (glucose-6-phosphatase) template: Glucose-6-Phosphatase PCR amplicon; primers: GSDw, GSDcom;

Figure 11a negative reaction, anodic sweep, peak position: 429mV, peak height: 1.84nA.

Figure 11b positive reaction (PCR amplicon template), anodic sweep, peak position: 388mV, peak height: 7.62nA.

Figure 11c positive reaction (genomic template), anodic sweep, peak position: 409mV, peak height: 8.11nA.

Example 5(c)

Oligonucleotide: T1BAPR oligonucleotide labelled at 5' end with a 9-carbon spacer moiety.

Positive reaction: (β actin) template: human genomic DNA; primers:

BAF, BAR.

Negative reaction: (glucose-6-phosphatase) template: human genomic DNA; primers: GSDw, GSDcom.

Voltammetry conditions: as in Table 1.

The results are as follows:

Figure 12a: negative reaction, anodic sweep.

Figure 12b: positive reaction, anodic sweep, peak position: 429mV, peak height: 36nA.

Figure 12c: negative reaction cathodic sweep.

Figure 12d: positive reaction cathodic sweep, peak position: 498mV, peak height: 14nA.

Example 5(d)

Oligonucleotide GSDPR labelled at 5′ end with a 12 carbon spacer moiety.

Positive reaction: (glucose-6-phosphatase) template: human genomic DNA; primers: GSDw, GSDcom.

Negative reaction: (β actin) template: human genomic DNA; primers: BAF, BAR.

Figure 13a: negative reaction, anodic sweep.

Figure 13b: positive reaction, anodic sweep, peak, position: 439mV, peak height: 23nA.

Figure 13c: negative reaction cathodic sweep.

Figure 13d: positive reaction cathodic sweep.

In this example, to demonstrate the sequence of specific detection of PCR products with ferrocenylated oligonucleotide probes, probe and primer sequences from previously optimized fluorogenic 5′ nuclease assays were used. PCR amplification from beta actin glucose-6-phosphatase and medium chain acyl-CoA dehydrogenase genes was performed using either purified amplicon or human genomic DNA template. In all PCR experiments probes with C12 ferrocene linkers attached at the 5′ end were used. The 3′ end of all PCR experiments probes were extension blocked by phosphorylation.

Ferrocenyl oligonucleotide probes were added to PCR mixes which amplified complementary targets (positive reactions) and non-complementary targets (negative reactions). To improve detection of the ferrocene

species, reactions were combined and concentrated before voltammetric analysis.

Voltammetric analysis was performed on the crude PCR mixes (Figures 10 and 11). In each case a ferrocene related signal is observed for positive reactions (containing digested probe). No signal is observed for negative reactions (containing undigested probe).

Claims

1. A method of probing for a nucleic acid comprising: contacting a nucleic acid solution with an oligonucleotide probe labelled with an electrochemically active marker, providing conditions at which the probe is able to at least partially hybridise with any complementary (target) sequence which may be present in the nucleic acid solution, selectively degrading either hybridised, partially hybridised or unhybridised nucleic acid probe, and electrochemically determining information relating to the electrochemically active marker.

2. A method as claimed in claim 1 wherein the information relating to the marker is used to derive information concerning the presence or absence of at least one nucleic acid species.

3. A method as claimed in claim 1 or claim 2 wherein the electrochemical technique is used to quantify relative proportions of degraded and non-degraded probe.

4. A method as claimed in any one of claims 1 to 3 wherein nucleic acid probe that has failed to successfully hybridise is digested by an enzyme that has been chosen to selectively digest single stranded (unhybridised) nucleic acid.

5. A method as claimed in claim 4 wherein the enzyme is an endonuclease.

6. A method as claimed in claim 4 or claim 5 wherein the enzyme is a ribonuclease.

7. A method as claimed in claim 4 or claim 5 wherein the enzyme is a deoxyribonuclease.

8. A method as claimed in any one of claims 4 to 7 wherein the enzyme is S1 deoxyribonuclease.

9. A method as claimed in any one of claims 1 to 3 wherein nucleic acid probe that has successfully hybridised is digested by an enzyme that has been chosen to selectively digest at least one strand of double stranded (hybridised) nucleic acid.

10. A method as claimed in claim 9 wherein the enzyme is a 5′ nuclease.

11. A method as claimed in claim 10 wherein the 5′ nuclease is also a DNA polymerase.

12. A method as claimed in claim 11 wherein the 5′ nuclease/ DNA polymerase is a thermostable enzyme.

13. A method as claimed in claim 12 wherein the thermostable enzyme is *Taq* polymerase.

14. A method as claimed in claim 12 or claim 13 wherein the reaction mixture also comprises a pair of primers suitable for extension by the DNA polymerase.

15. A method as claimed in claim 14 wherein reaction conditions and temperature cycling are suitable for a polymerase chain reaction (PCR) to take place concomitant to the 5′ nuclease digestion of probe.

16. A method as claimed in any one of claims 1 to 3, in which a first oligonucleotide probe labelled with an electrochemically active marker is prevented from complete hybridisation by competition from a second oligonucleotide, and the resultant partially hybridised oligonucleotide labelled with an electrochemically active marker is cleaved by an enzyme that specifically recognises the configuration of the two oligonucleotides hybridised onto the target nucleic acid, said cleavage effectively shortening the oligonucleotide portion to which the electrochemically active marker is attached.

17. A method as claimed in any one of claims 1 to 3, in which a first oligonucleotide probe is prevented from complete hybridisation by competition from a second oligonucleotide, and the resultant partially hybridised first oligonucleotide probe is cleaved by an enzyme that specifically recognises the configuration of the two oligonucleotides hybridised onto the target nucleic acid, the cleavage product being recognised by a recognition cassette which comprises at lease one oligonucleotide and is able to hybridise to the first cleavage product to produce an oligonucleotide configuration recognisable by an enzyme that cleaves a region of the recognition cassette that is labelled with an electrochemically active marker.

18. A method as claimed in any one of the preceding claims for the detection of nucleic acid polymorphisms.

19. A method as claimed in any one of the preceding claims for detection of allelic polymorphisms.

20. A method as claimed in any one of the preceding claims for the detection of single nucleotide polymorphisms.

21. A method as claimed in any one of claims 1 to 17 for the quantification of nucleic acid species.

22. A method as claimed in any one of claims 1 to 17 for the quantification of gene expression.

23. A method as claimed in any one of claims 14 to 22 wherein primer design and/or probe design and/or thermal cycling and detection of electrochemically active marker is carried out automatically or with the assistance of a software-directed microprocessor.

24. A method of detecting a specific protein or group of proteins, comprising: contacting a protein solution with an oligonucleotide probe labelled with an electrochemically active marker, providing conditions at which the probe is able to bind to any specific protein or group of proteins that may be present in the solution, selectively degrading unhybridised nucleic acid probe, and electrochemically determining information relating to the electrochemically active marker in order to provide information about the presence, absence or relative or absolute amounts of the specific target protein or group of target proteins present in said solution.

25. A method as claimed in claim 24 wherein the oligonucleotide probe sequence is substantially similar to an *in vivo* protein recognition site and the protein or group of proteins potentially detected would ordinarily be regarded as a nucleic acid binding protein(s).

26. A method as claimed in claim 24 wherein the oligonucleotide probe comprises an aptamer which has been selected to bind to a specific protein or group of proteins.

27. A method as claimed in any one of claims 22 to 26 wherein the unhybridised nucleic acid is degraded (digested) by an enzyme.

28. A method as claimed in claim 27 wherein the enzyme is an endonuclease.

29. A method as claimed in claim 27 or claim 28 wherein the enzyme is a ribonuclease.

30. A method as claimed in any one of claims 27 to 29 wherein the enzyme is a deoxyribonuclease.

31. A method as claimed in any one of claims 27 to 30 wherein the enzyme is S1 deoxyribonuclease.

32. A method as claimed in any one of claims 24 to 31 for the detection of protein polymorphisms.

33. A method as claimed in any one of claims 24 to 32 for the quantification of protein expression.

34. Use of a method as claimed in any one of the preceding claims in the detection of a genetic disease or a genetic disease carrier status or a genetic predisposition to disease.

35. Use of a method as claimed in any one of the preceding claims to detect or identify a pathogen in a sample.

36. Use of a method as claimed in any one of claims 1 to 33 to predict a response of an organism to a therapeutic or toxic agent.

37. A method as claimed in any one of the preceding claims wherein the electrochemical method is voltammetry.

38. A method as claimed in any one of claims 1 to 36 wherein the electrochemical technique is an amperometric technique.

39. A method as claimed in claim 37 claims wherein the method used is differential pulse voltammetry.

40. A method as claimed in any of the preceding claims wherein the electrochemical technique utilizes one or more electrodes that have been functionally surrounded by a selectively permeable membrane.

41. A method as claimed in claim 40 wherein the membrane is selectively permeable on the basis of molecular size.

42. A method as claimed in claim 40 or claim 41 wherein the membrane is selectively permeable on the basis of charge.

43. A method as claimed in any one of claim 40 to 42 wherein the membrane is selectively permeable on the basis of hydrophobicity or hydrophilicity.

44. A nucleic acid probe molecule comprising an oligonucleotide of specific sequence covalently linked to one or more electrochemically active marker moieties.

45. A probe as claimed in claim 44 wherein one or more electrochemically active marker moieties are linked to the oligonucleotide via a linker comprising an aliphatic chain having at least 4 carbon atoms.

46. A probe as claimed in claim 44 or claim 45, which comprises at least one metallocene moiety.

47. A probe as claimed in any one of claims 44 to 46, which comprises at least one ferrocene moiety.

48. A probe as claimed in anyone of claims 44 to 47 wherein the oligonucleotide component is optimised in terms of length or sequence to hybridise to a target nucleic acid sequence.

49. A probe as claimed in any one of claims 44 to 49 wherein the oligonucleotide component is optimised in order to hybridise to a target DNA sequence at a position intermediate between a matched pair of oligonucleotide PCR primers, so that upon primer extension the oligonucleotide component of the probe may be digested by a 5′ nuclease activity of the thermostable DNA polymerase.

50. A probe as claimed in any one of claims 44 to 48 wherein the oligonucleotide component is optimised in order to partially hybridise to a target nucleic acid sequence at a position which overlaps with a second hybridised oligonucleotide, the overlap region being

situated towards the 5′ end of the probe, said 5′ end being prevented from complete hybridisation to the target nucleic acid by the presence of the second oligonucleotide.

51. A probe as claimed in any of claims 44 to 48 wherein said probe is a recognition cassette labelled with an electrochemically active marker and optimised to hybridise to a target nucleic acid sequence so as to form a region of nucleic acid triplex which can be specifically recognised by an enzyme, said recognition resulting in cleavage of said recognition cassette.

52. A probe as claimed in any one of claims 44 to 47 wherein the nucleic acid component is optimised in terms of length or sequence to hybridise to a target protein.

53. A probe as claimed in claim 52 wherein the probe comprises an aptamer.

54. A probe claimed in claim 52 or claim 53 wherein the probe substantially comprises the nucleic acid sequence of a naturally occurring protein recognition site.

55. A probe as claimed in any one of claims 44 to 54 wherein an electrochemically active marker is attached to the 3′ end of the oligonucleotide probe.

56. A probe as claimed in any one of claims 44 to 55 wherein an electrochemically active marker is attached to the 5′ end of the oligonucleotide probe.

57. A probe as claimed in any one of claims 44 to 56 wherein multiple electrochemically active markers are attached along the length of the oligonucleotide probe.

58. A probe as claimed in any one of claims 44 to 57 wherein an electrochemically active marker is attached to substantially all of nucleotide residues of the oligonucleotide probe.

59. A probe as claimed in any one of claims 44 to 58 wherein one of more electrochemically active marker moiety is as according to formula I, II, III or IV.

60. An oligonucleotide probe substantially as described in any of examples 3, 4a to 4h, 5a to 5c, 14a and 14b.

61. A probe as claimed in any one of claims 44 to 60 wherein the oligonucleotide component is phosphorylated at both the 3′ and 5′ ends.

62. A kit comprising an oligonucleotide labelled with an electrochemically active marker and any one or more other component such as oligonucleotide primers or enzymes optimised for use with the labelled oligonucleotide in accordance with any of the preceding method or use claims.

63. A kit as claimed in claim 62, comprising an oligonucleotide probe labelled with an electrochemically active marker and S1 nuclease.

64. A kit as claimed in claim 62, comprising an oligonucleotide probe and a pair of PCR primers.

65. A kit as claimed in claim 62 or claim 64, comprising a nucleic acid polymerase that exhibits a 5′ nuclease activity.

66. Apparatus arranged to carry out any one or more of method claims 1 to 33 or 37 to 43.

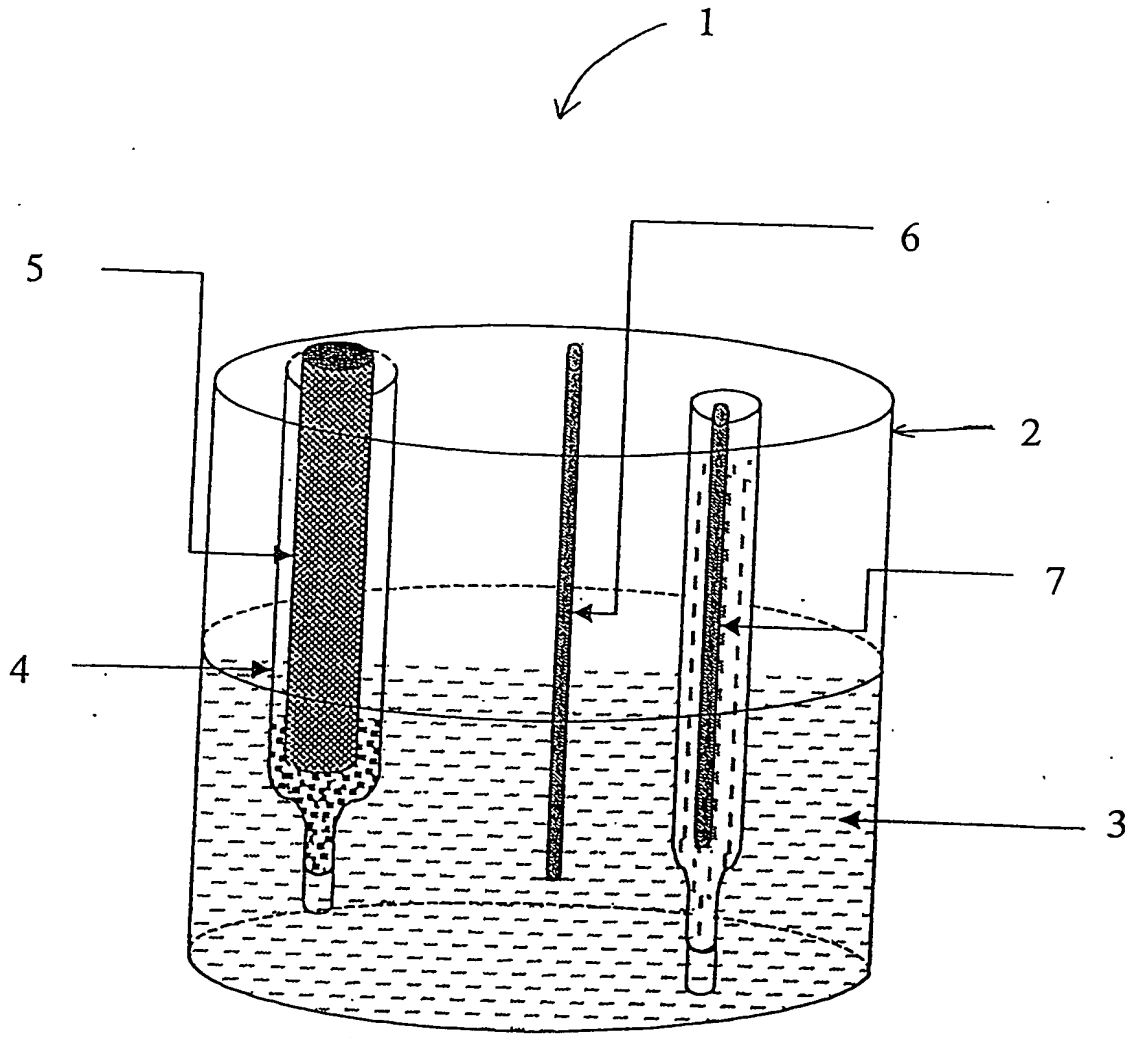


Fig. 1

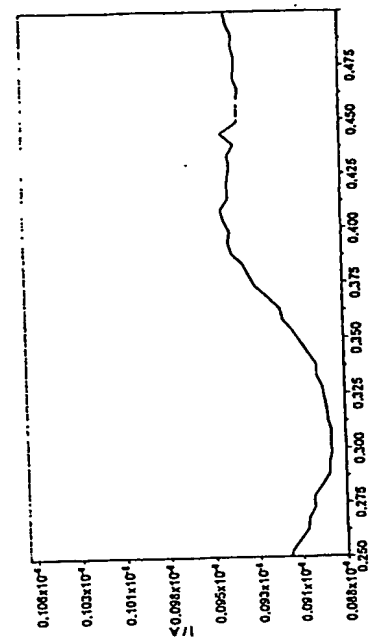


Fig. 2c

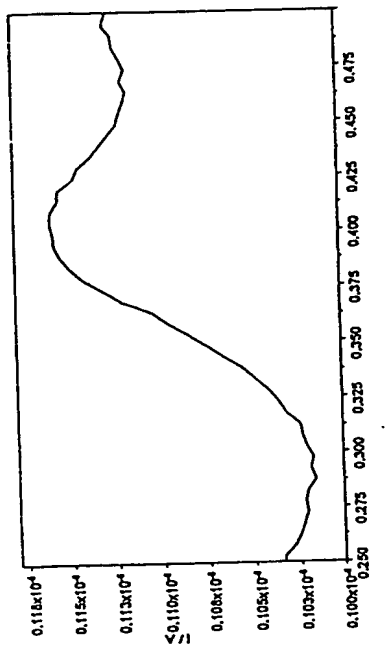


Fig. 2d

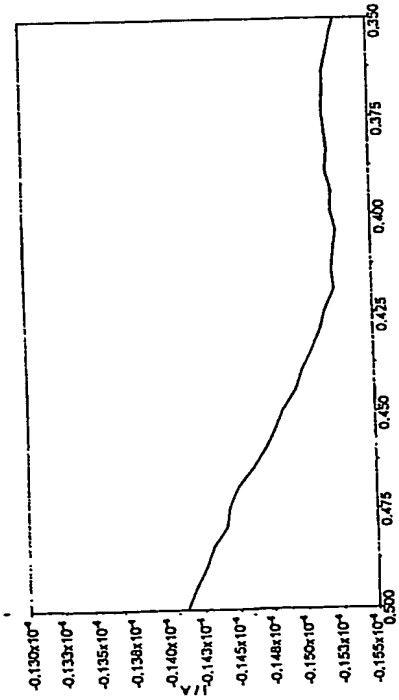


Fig. 2a

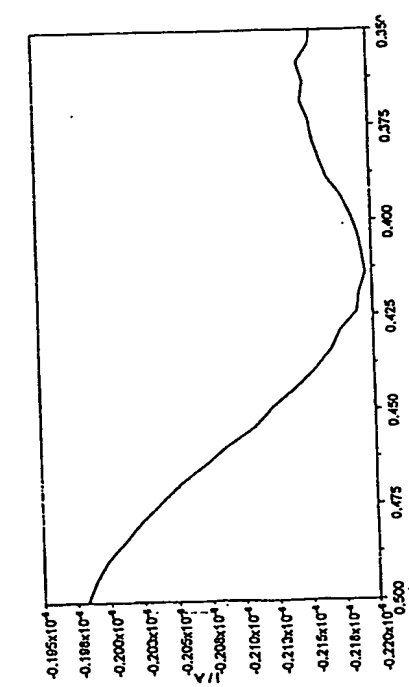


Fig. 2b

Fig. 2

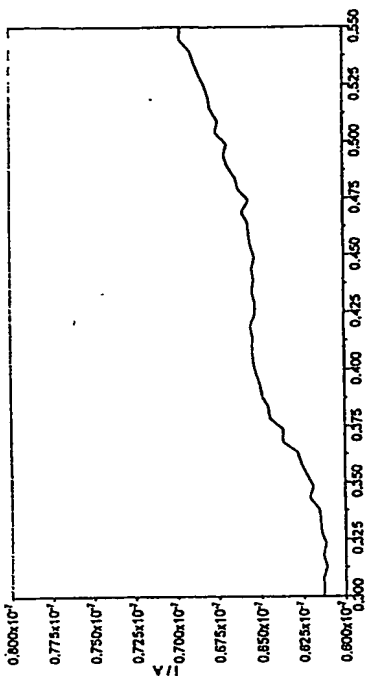


Fig. 3c

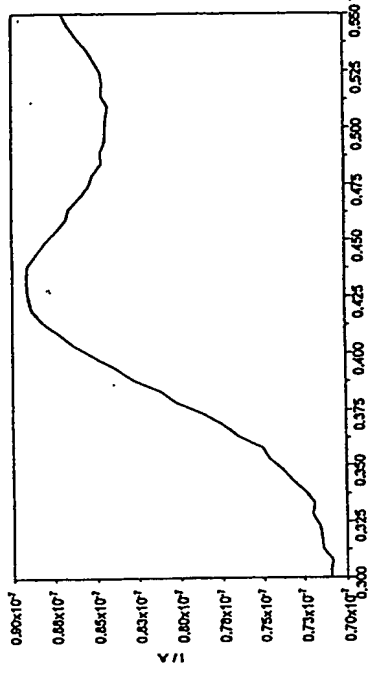


Fig. 3d

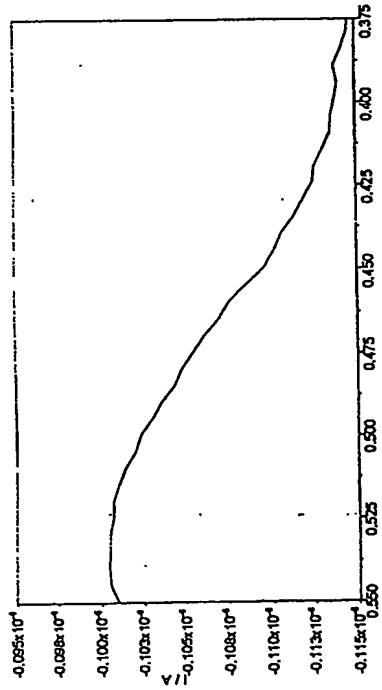


Fig. 3a

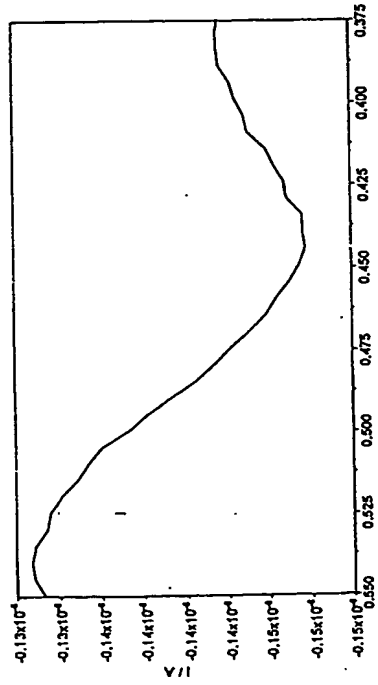


Fig. 3b

Fig. 3

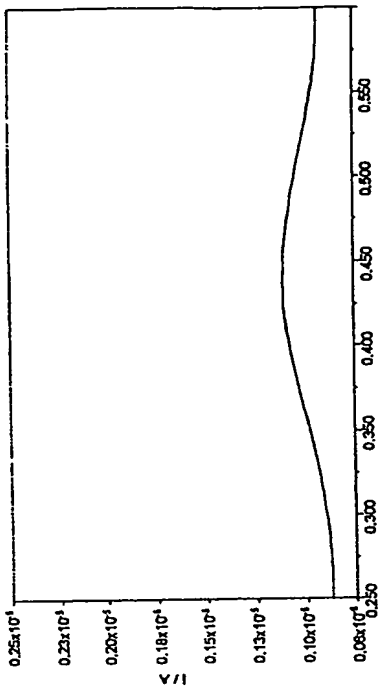


Fig. 4c

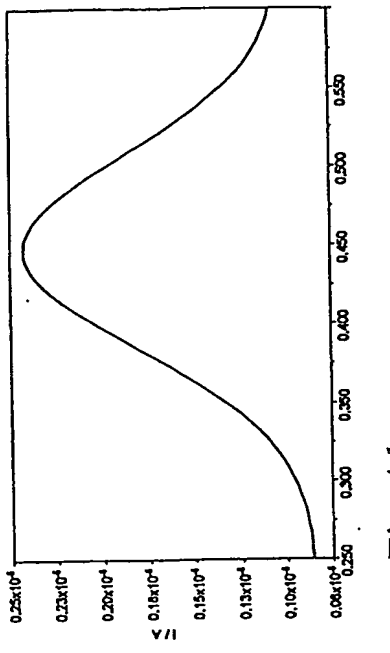


Fig. 4d

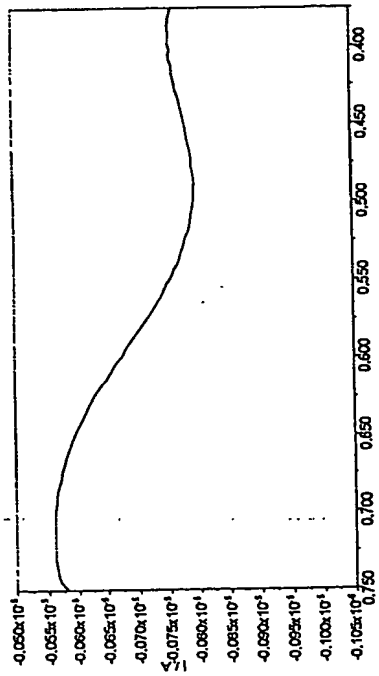


Fig. 4a

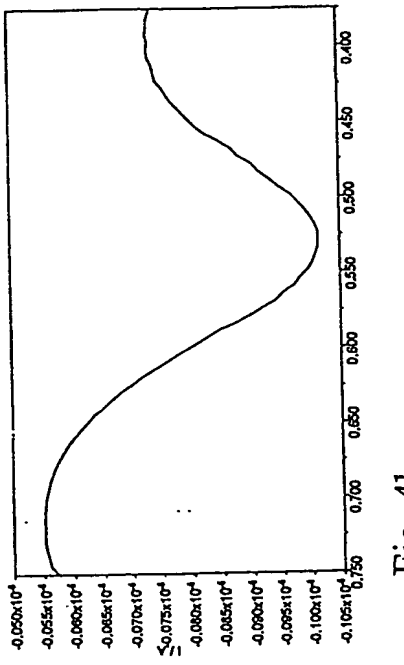


Fig. 4b

Fig. 4

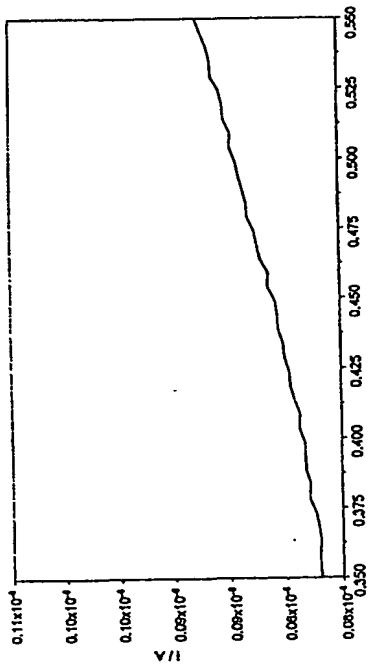


Fig. 5c

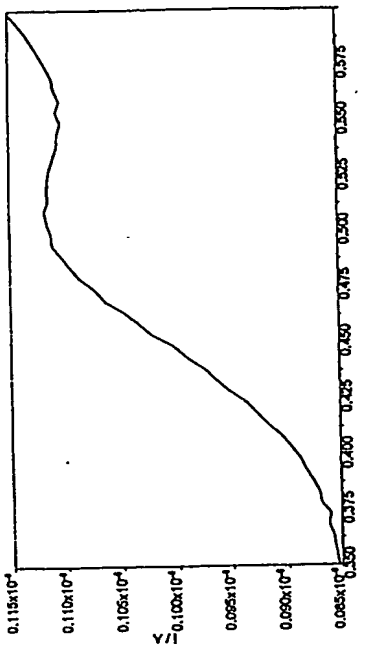


Fig. 5d

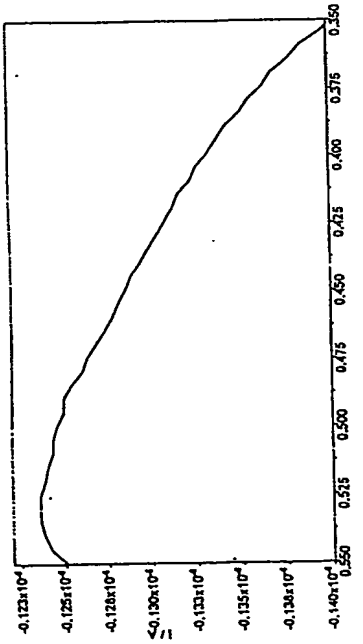


Fig. 5a

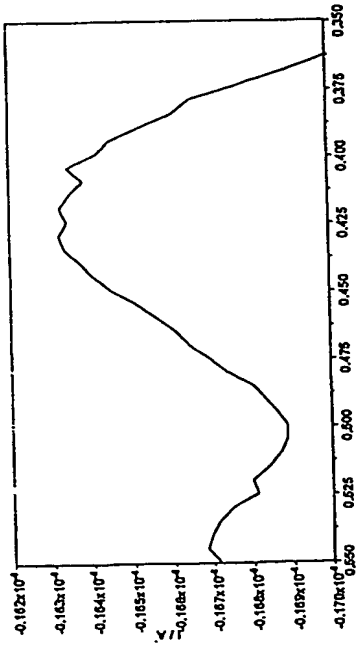


Fig. 5b

Fig. 5

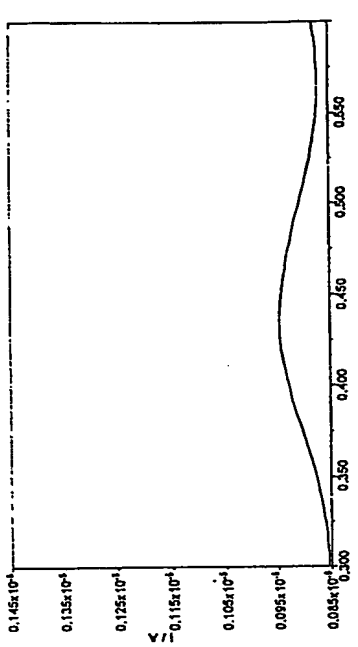


Fig. 6c

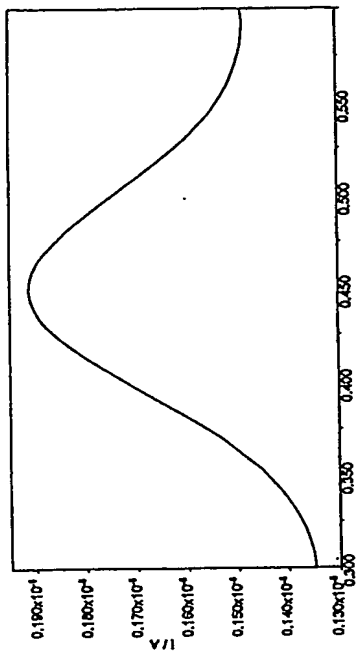


Fig. 6d

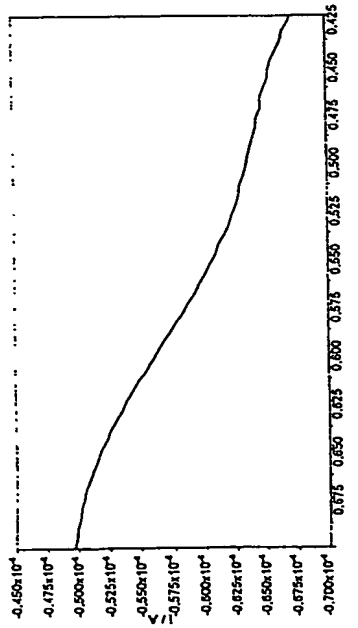


Fig. 6a

Fig. 6b

Fig. 6

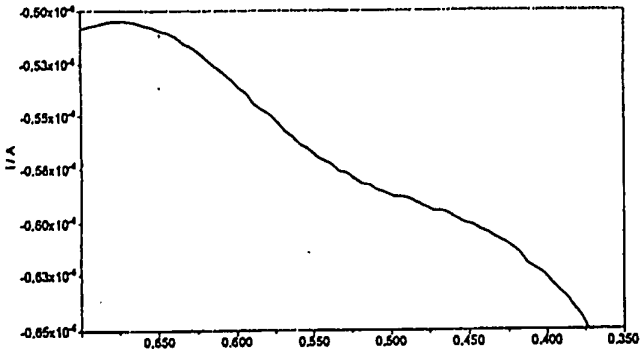


Fig. 7a

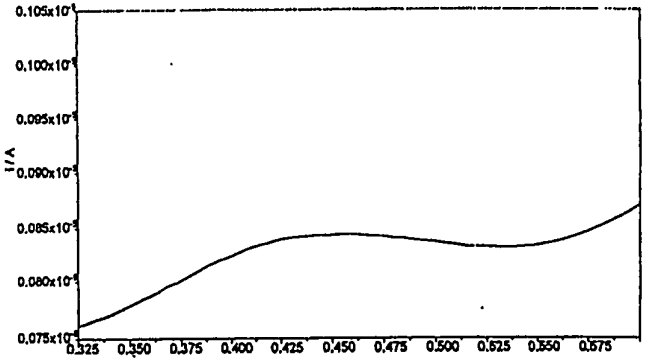


Fig. 7c

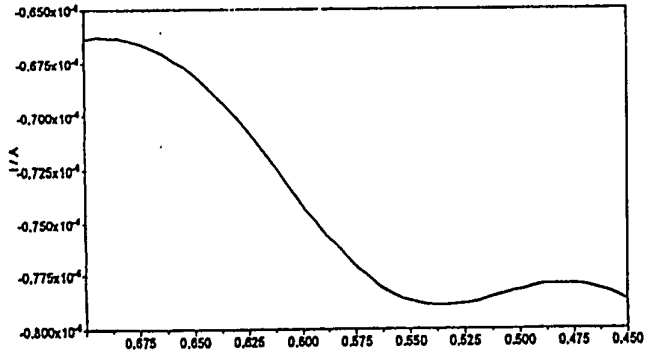


Fig. 7b

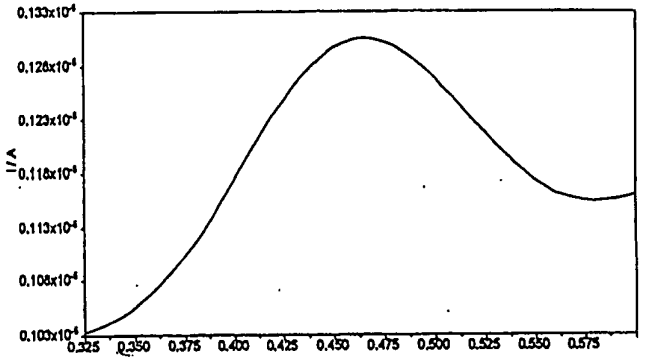


Fig. 7d

Fig. 7

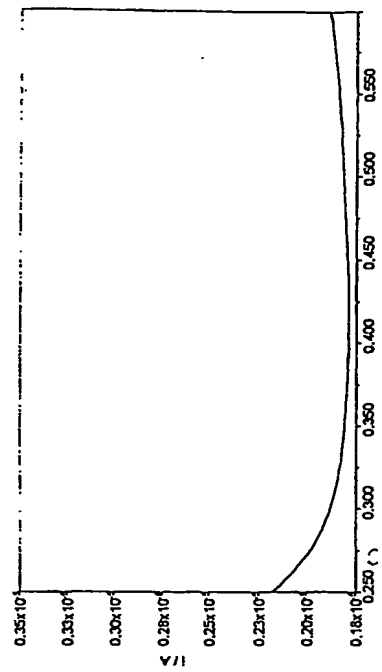


Fig. 8b

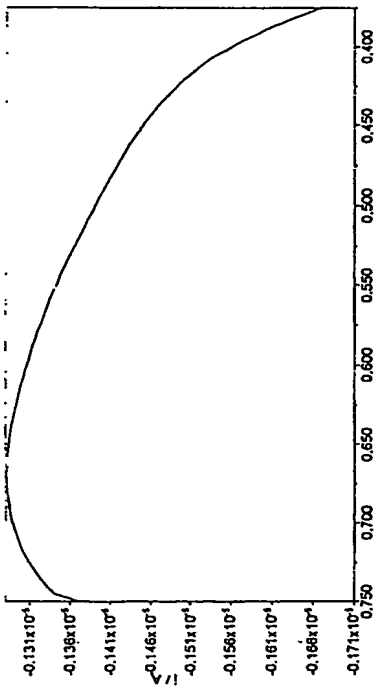


Fig. 8a

Fig. 8

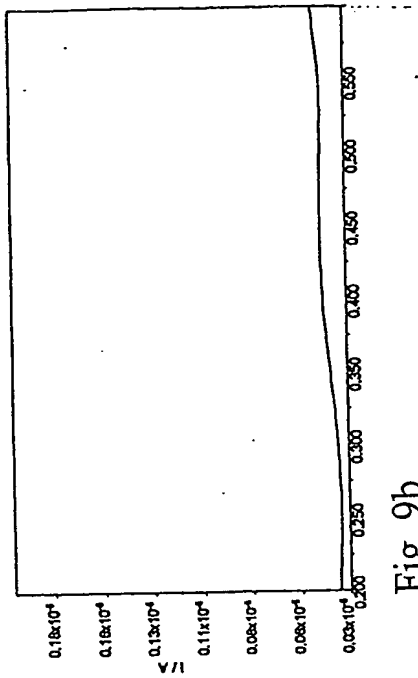


Fig. 9b

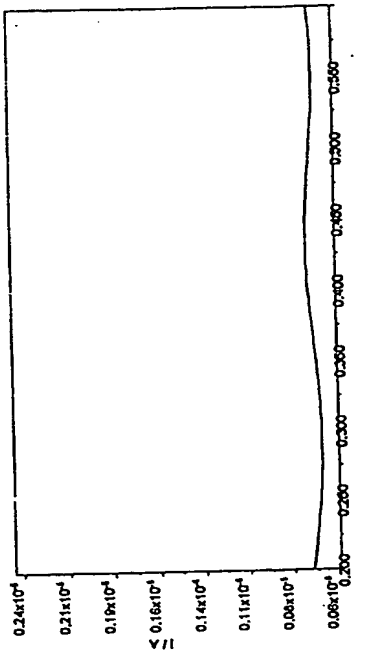


Fig. 9a

Fig. 9

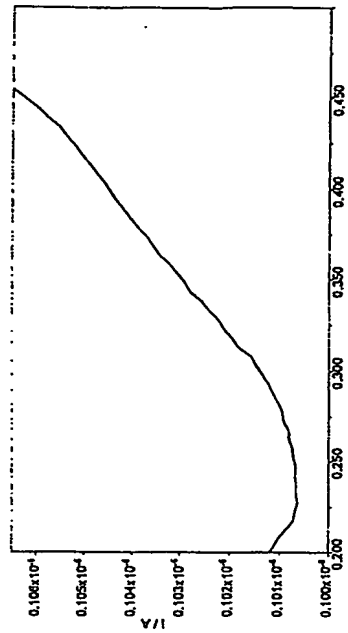


Fig. 10c

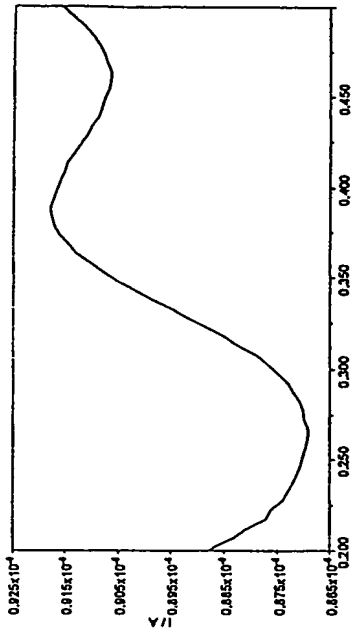


Fig. 10d

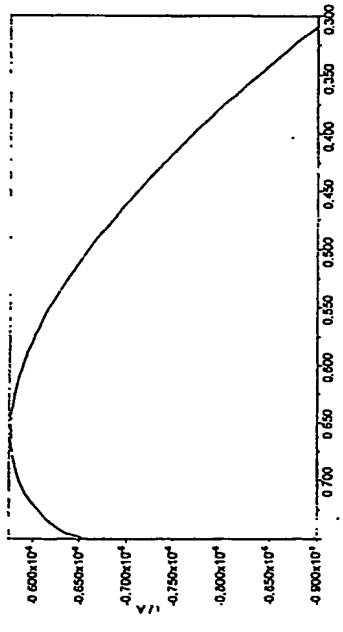


Fig. 10a

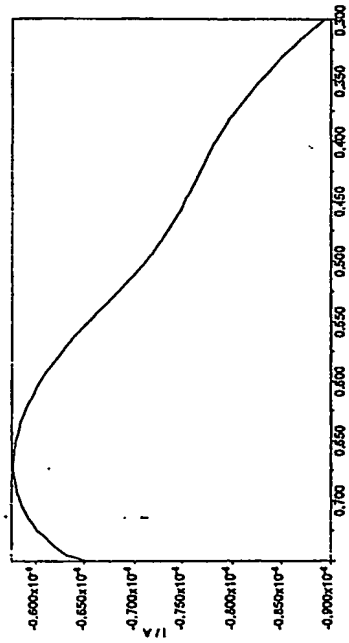


Fig. 10b

Fig. 10

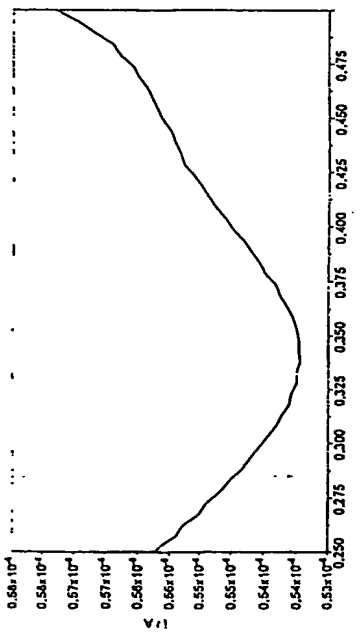


Fig. 11a

Fig. 11b

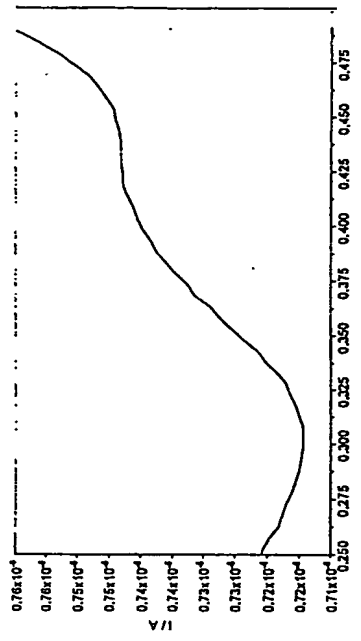


Fig. 11c

Fig. 11

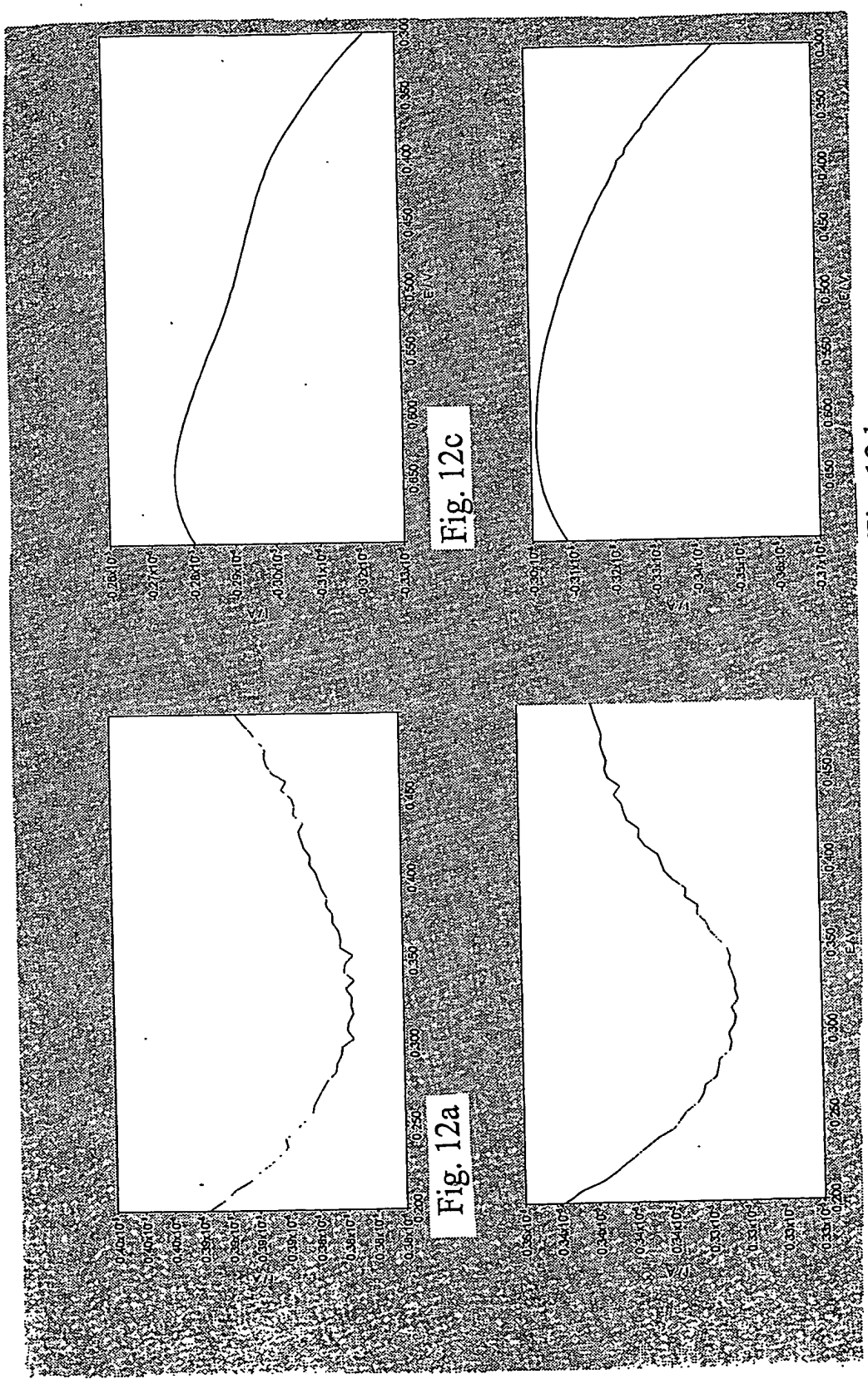


Fig. 12a

Fig. 12b

Fig. 12c

Fig. 12d

Fig. 12

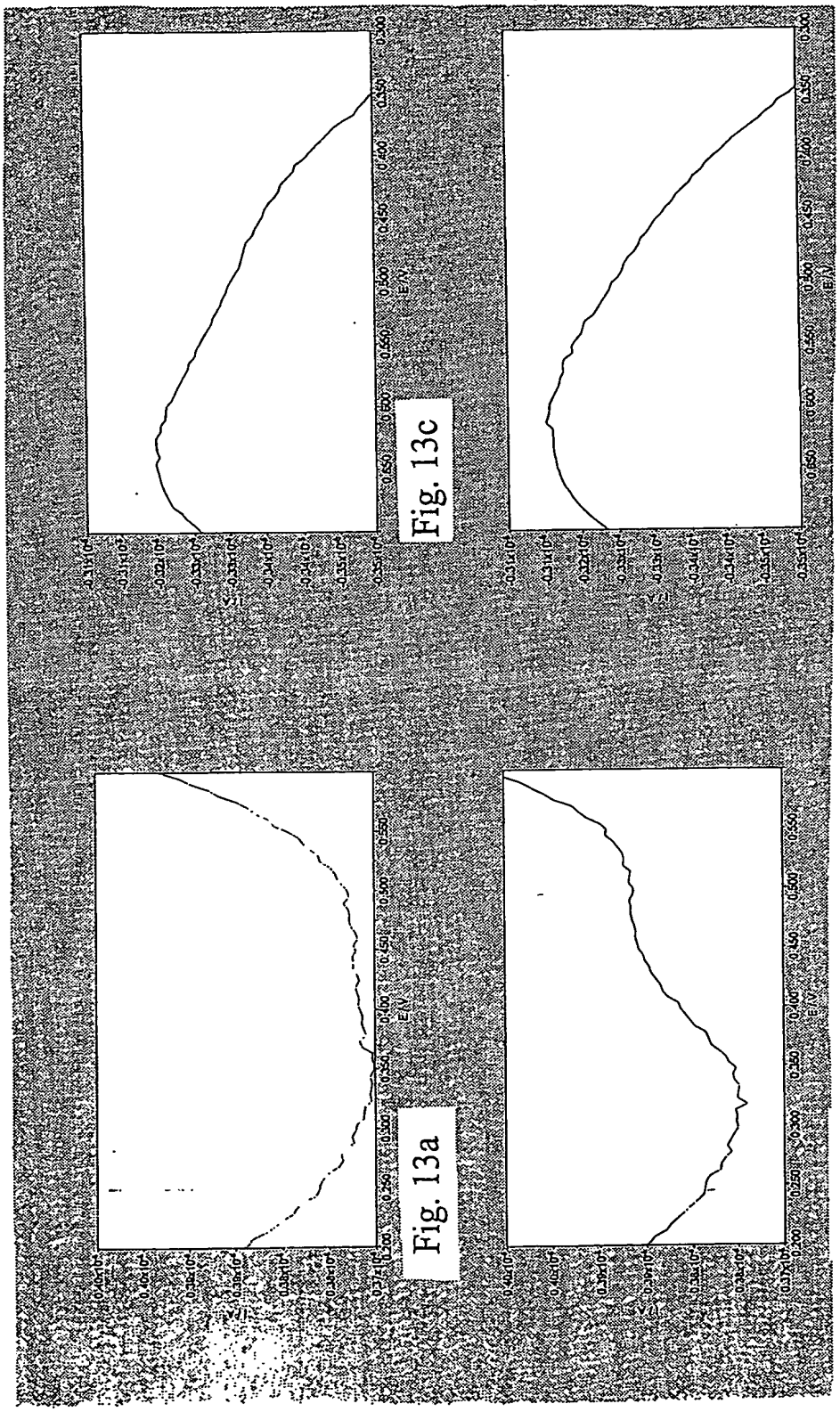


Fig. 13a

Fig. 13b

Fig. 13c

Fig. 13d

Fig. 13

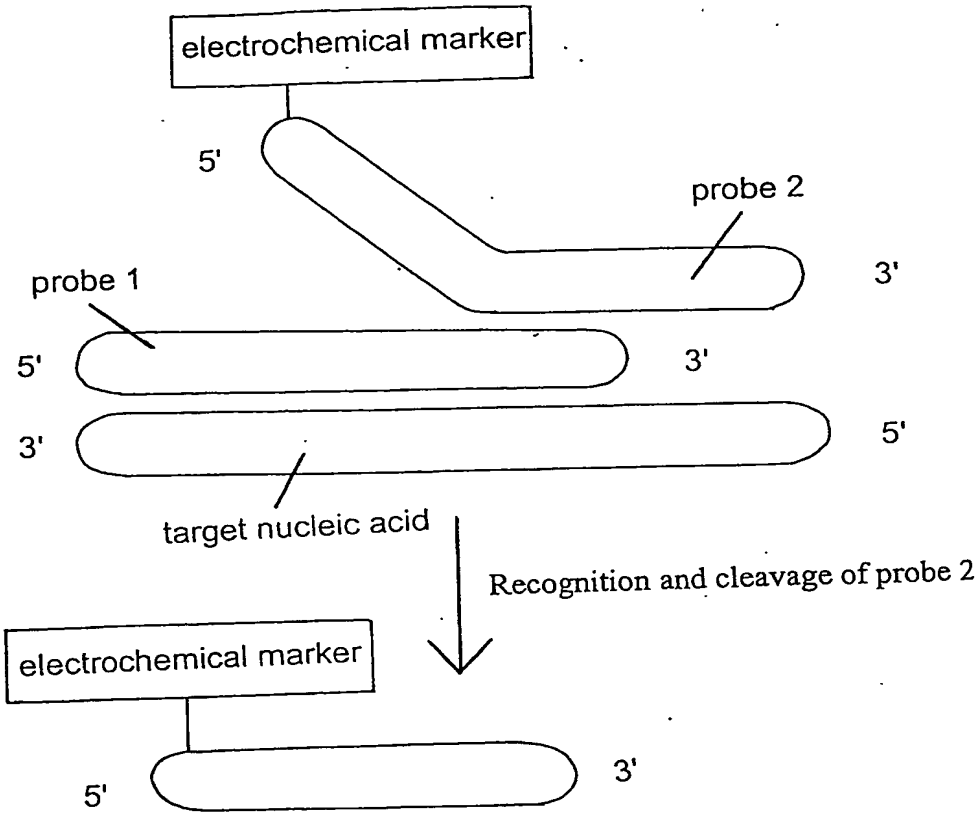

electrochemical marker
5'
probe 2
3'
probe 1
5'
3'
3'
5'
target nucleic acid
Recognition and cleavage of probe 2
electrochemical marker
5'
3'

Fig. 14a

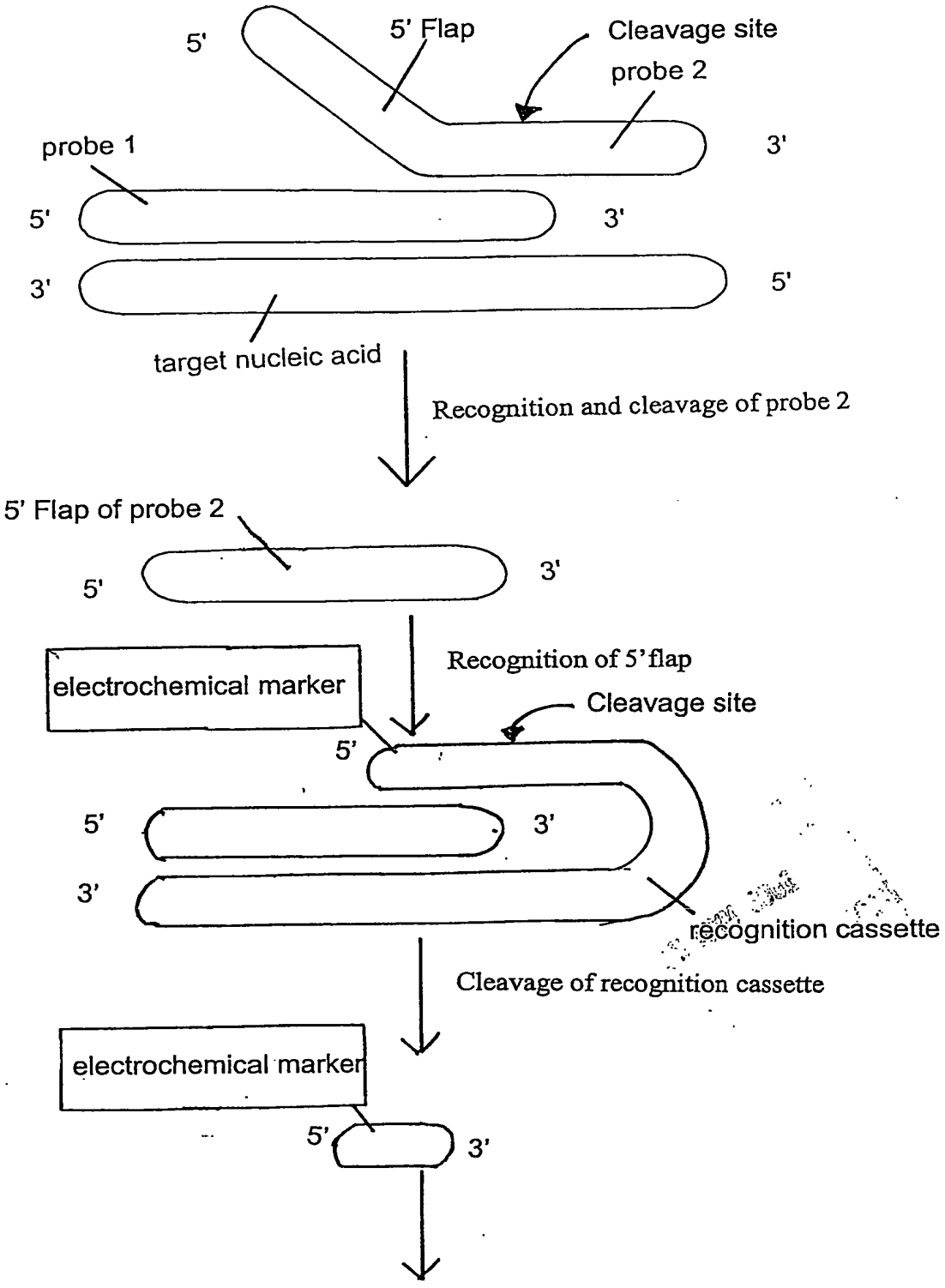
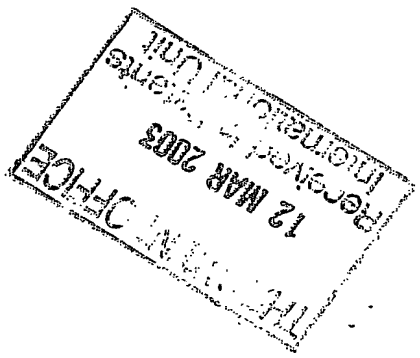

5'
5' Flap
Cleavage site
probe 2
3'
probe 1
5'
3'
3'
5'
target nucleic acid
Recognition and cleavage of probe 2
5' Flap of probe 2
5'
3'
Recognition of 5'flap
electrochemical marker
Cleavage site
5'
5'
3'
3'
recognition cassette
Cleavage of recognition cassette
electrochemical marker
5'
3'
Signal (change in electrochemical activity of marker)

Fig. 14b


THE PATENT OFFICE
12 MAR 2003